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RESEARCH PAPER

The use of metabolomics integrated with transcriptomic and proteomic studies for identifying key steps involved in the control of nitrogen metabolism in crops such as maize

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Abstract

Linking plant phenotype to gene and protein expression and also to metabolite synthesis and accumulation is one of the main challenges for improving agricultural production worldwide. Such a challenge is particularly relevant to crop nitrogen use efficiency (NUE). Here, the differences in leaf gene transcript, protein, and metabolite accumulation in maize subjected to long-term nitrogen (N)-deficient growth conditions at two important stages of plant development have been studied. The impact of N deficiency was examined at the transcriptomic, proteomic, and metabolomic levels. It was found that a number of key plant biological functions were either up- or down-regulated when N was limiting, including major alterations to photosynthesis, carbon (C) metabolism, and, to a lesser extent, downstream metabolic pathways. It was also found that the impact of the N deficiency stress resembled the response of plants to a number of other biotic and abiotic stresses, in terms of transcript, protein, and metabolite accumulation. The genetic and metabolic alterations were different during the N assimilation and the grain-filling period, indicating that plant development is an important component for identifying the key elements involved in the control of plant NUE. It was also found that integration of the three 'omics' studies is not straightforward, since different levels of regulation seem to occur in a stepwise manner from gene expression to metabolite accumulation. The potential use of these 'omics' studies is discussed with a view to improve our understanding of whole plant nitrogen economics, which should have applications in breeding and agronomy.

Key words: Assimilation, kernel filling, maize, metabolome, nitrogen, proteome, remobilization, transcriptome, yield.

Introduction

Over the last four decades, the rate of crop yield improvement has accelerated exponentially, primarily due to the introduction of an increasingly scientific approach to plant breeding using molecular markers, but also through the extensive use of mineral fertilizers

Abbreviations: C, carbon; M, mature stage; N, nitrogen; N⁺, high nitrogen non-limiting conditions; N⁻, low nitrogen-deficient conditions; NUE, nitrogen use efficiency; V, vegetative stage.

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during the green revolution (Tilman *et al.*, 2002). Among these mineral fertilizers, nitrogen (N) has been the major limiting factor in agricultural production (Vitousek *et al.*, 1997) and the greatest input cost for a large number of crops. However, this extensive use of N fertilizers has had a serious detrimental impact on the diversity and functioning of the non-agricultural bacterial, animal, and plant ecosystems, as well as on the composition of the atmosphere (Galloway *et al.*, 2003, 2008). Between now and 2025, the human population is expected to increase from 7 to 9 billion people. Thus, the challenge for the next decades will be to accommodate the needs of the expanding world population by developing highly productive agriculture, while at the same time preserving the quality of the environment by rationalizing the use of N fertilizers (Dyson, 1999). Therefore, improving N use efficiency (NUE) is particularly relevant to most crops requiring large amounts of N fertilizers to obtain maximum yield, since NUE has been estimated on average to be far less than 50% (Raun and Johnson, 1999). NUE can be defined as the grain or biomass yields per unit of available N in the soil, which includes the residual mineral nutrients, the organic N present in the soil, and that provided by fertilization. Thus, an increasing number of studies combining whole plant physiology, molecular genetics, and agronomy have been undertaken over the last two decades to obtain a better understanding of the physiological and molecular basis of plant NUE, with a particular emphasis on crops such as rice, wheat, and maize (Hirel *et al.*, 2007). Among these studies, those using ‘omics’-based techniques have been extensively developed to obtain an overview of the response of plants to various growth and environmental conditions and in particular abiotic and biotic stresses. Metabolomic, transcriptomic, and, to a lesser extent, proteomic studies have been conducted for the high-throughput phenotyping necessary for large-scale physiological, molecular, and quantitative genetic studies, aimed at identifying the function of a particular gene, or set of genes involved in the control of complex physiological traits such as NUE (Meyer *et al.*, 2007; Liseć *et al.*, 2008; Kusano *et al.*, 2011). Although, the ‘omics’ approach provides a static picture of the physiological status of a given organ, tissue, or even cellular compartment (Tohge *et al.*, 2011) at a particular stage of plant development, it also provides information on changes in the concentration of an entire range of metabolites, proteins, and gene transcripts (Barhman *et al.*, 2004; Krapp *et al.*, 2007; Saito *et al.*, 2007).

In order to improve our knowledge of the physiological and molecular responses of maize leaves to long-term N deprivation, a metabolomic analysis was conducted in parallel with a proteomic and a transcriptomic study at two key stages of plant development. These studies provide an integrated view of the adaptive responses of the plant to prolonged N stress at three important biological levels, spanning from gene expression to metabolite accumulation. Whether these traits can be used as investigation tools to monitor N fertilization or to identify candidate genes for NUE improvement is discussed.

Materials and methods

Plant material

Maize (*Zea mays* L., genotype B73) seeds were first sown on coarse sand and, after 1 week, when 2–3 leaves had emerged, individual plants

were transferred to pots (diameter and height of 30 cm) containing clay loam soil with one plant per pot and 12 pots in total. Clay loam soil is composed of a mixture of loam (washed fine silt with no minerals) and loam balls of ~0.5 cm diameter that ensure sufficient aeration of the roots and allow the growth of the plant until maturity without lodging. Clay loam soil also allows a constant flow of the nutrient solution that is provided several times a day. Pots were placed in a glasshouse at the Institut National de la Recherche Agronomique, Versailles, France and grown from May to September 2004. Pots were moved every week to avoid shading and position effects.

Three individual plants of similar size and of similar developmental stage were randomly selected. They correspond to the three replicates used for the three different ‘omics’ experiments. At the 10- to 11-leaf stage, the three youngest fully expanded leaves were harvested and pooled for the vegetative stage (V) samples to obtain enough homogenous plant material representative of this plant developmental stage. The leaf below the ear was harvested at 55 days after silking (DAS) and was named the mature leaf developmental stage (M). The M stage corresponds to the end of the grain-filling period, during which, under the experimental conditions used here, leaves did not show any visible symptoms of yellowing. For the measurement of markers for plant N status, the leaf below the emerging ear 15 DAS was harvested (F stage). Harvesting of the ear was performed ~80 d after flowering. The leaf, below the ear, was selected since it has been shown to provide a good indication of the source–sink transition during kernel filling (Hirel *et al.*, 2005; Martin *et al.*, 2005). Leaf samples were harvested between 09:00 h and 12:00 h, frozen in liquid N₂, ground to a homogenous powder, and stored at –80 °C. Choosing a single time point during the middle of the light period is commonly used in a large number of physiological and molecular studies and was successfully used in a previous quantitative genetic study (Gallais and Hirel, 2004). In the glasshouse, plants were watered daily with a complete nutrient solution (N⁺) containing 10 mM KNO₃ as the sole N source (Coïc and Lesaint, 1971). The complete nutrient solution contained 1.25 mM K⁺, 0.25 mM Ca²⁺, 0.25 mM Mg²⁺, 1.25 mM H₂PO₄⁻, 0.75 mM SO₄²⁻, 21.5 mM Fe²⁺ (Sequestrene; Ciba-Geigy), 23 mM B³⁺, 9 mM Mn²⁺, 0.3 mM Mo²⁺, 0.95 mM Cu²⁺, and 3.5 mM Zn²⁺. For growing plants under low N-deficient conditions (N⁻), NO₃⁻ was supplied as 0.1 mM KNO₃, an N concentration that has previously been shown to provide N deficiency stress for most plant species (Hirel *et al.*, 2005; Tercé-Laforgue *et al.*, 2005).

RNA preparation

Total RNA was extracted as described by Verwoerd *et al.* (1989) from leaves that had been stored at –80 °C. A 50 mg aliquot of total RNA was incubated at 37 °C for 30 min with 40 U of RNase inhibitor and 25 U of RNase-free DNase (Promega, Charbonnières, France) in 6 ml of 10× buffer (Promega) with diethylpyrocarbonate (DEPC)-treated water added to a final volume of 60 ml. The DNase was removed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction, and total RNA was precipitated overnight at –20 °C in 0.1 vol. of ammonium acetate (3 M) and 2.5 vol. of ethanol (100%), and resuspended in DEPC-treated water. Purification of mRNA from total RNA was performed using the Dynabeads mRNA Direct Kit (Dyna/Invitrogen, Cergy Pontoise, France).

Gene expression profiles using maize cDNA microarrays

Starting with 3 µg of total RNA, non-modified amplified antisense RNA (aRNA) products were prepared using the Amino Allyl MessageAmpTM aRNA Kit (Ambion, Foster City, CA, USA) according to the manufacturer’s instructions. Briefly, RNA was transcribed into cDNA using reverse transcriptase with a T7 primer that contains a promoter for DNA-dependent RNA polymerase (Eberwine, 1996). After RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA (dscDNA) was purified and served as a template in the subsequent *in vitro* transcription reaction. Following this, 2 µg aliquots of aRNA were labelled using the SuperSriptTM Indirect cDNA Labeling System Kit (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer’s protocol, except that the purification steps were carried out using QIAquick[®] PCR columns (QIAGEN, Hilden, Germany). The quantity

and quality of each intermediate product, including total RNA, dsDNA, aRNA, and labelled targets, were evaluated using a Nanodrop ND-1000 spectrophotometer and an Agilent Technologies 2100 Bioanalyzer (Imbeaud *et al.*, 2005). Whole genome leaf transcript profiling was performed using the maize 46K arrays obtained from the maize oligonucleotide array project (<http://www.maizearray.org/index.shtml>).

Transcript abundance in each of the three replicates for V and M leaves at low (N⁻) and high (N⁺) N supply was determined using a mixture of all the samples (12 in total, each with the same mRNA concentration) as a reference. Hybridizations between the maize oligonucleotide microarrays and fluorescently labelled samples were performed in MICROMAX Hybridisation Buffer III (Perkin Elmer) using the manufacturer's hybridization and wash conditions and a GeneTac™ HybStation (Genomic Solutions, Ann Arbor, MI, USA). Before hybridization, 50 pmol Cy3- and 50 pmol Cy5-labelled targets were mixed, dried using compressed air, and reconstituted with 115 µl of hybridization buffer, followed by denaturing at 90 °C for 3 min. Each hybridization mixture was placed on the maize 45K array slides mounted in the hybridization station and the hybridizations were performed for 3 h at 65 °C, followed by 3 h at 55 °C, then 12 h at 50 °C with gentle agitation. Thereafter, the arrays were automatically washed with the GeneTac™ washing solutions (Genomic Solutions) using the program for multiple automatic washes, with a flow time of 40 s. Immediately after the completion of the final washing step, the arrays were removed from the station, briefly immersed in distilled water, and air-dried with ozone-safe dry air.

Hybridized microarrays were scanned using a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA) at 10 µm resolution and variable photomultiplier voltage to obtain maximal signal intensities with <0.05% probe saturation. Subsequent image analysis was performed with the GenePix Pro (v6.0.1.26) software. Analysis included defining the spots, measuring the intensities, flagging spots when inadequate quality control parameters were found, and evaluating local background. The resulting files, containing all the scan data, were further processed using the statistical programming language R (<http://www.r-project.org>) together with the packages of the MAnGO project [Version 0.9.7, MicroArray Normalisation tool of GODMAP, CNRS BioInfo Team (<http://bioinfome.cgm.cnrs-gif.fr/>), (Marisa *et al.*, 2007)]. The background level was calculated using morphological operators (a short closing followed by a large opening) and subtracted. Raw data were normalized using a global loess method (Smyth and Speed, 2003). Gene annotation was provided by the maize oligonucleotide array project mentioned above (<http://www.maizearray.org/index.shtml>).

Statistical analysis of maize cDNA microarray data

Statistical group comparisons were performed using multiple testing procedures to evaluate statistical significance for differentially expressed genes. Two gene selection approaches were applied, including the significance analysis of microarrays (SAM; Tusher *et al.*, 2001) permutation algorithm, and a *P*-value ranking strategy using both *z*-statistics in ArrayStat 1.0 software (Imaging Research Inc.) and moderated *t*-statistics using a moderated *t*-test available in MAnGO tools (<http://bioinfome.cgm.cnrs-gif.fr/>) and BRBArrayTools v3.2.3 packages (Korn *et al.*, 2002). For multiple testing corrections, the false discovery rate (FDR) procedure was used (Benjamini and Hochberg, 1995). Statistical tests were computed and combined for each probe set using the log-transformed data, and a probe set was declared to be significant when the adjusted *P*-value was less than the effective α -level ($\alpha=0.05$) in at least one of these tests. A filtering procedure additionally excluded those data points considered biologically unreliable due to low signal intensities (Amean <7.0). Transcriptomic data were validated by qRT-PCR analysis performed on a selected number of up- or down-regulated gene transcripts (data not shown).

Total protein extraction, solubilization, and quantification

A trichloroacetic acid/acetone protein precipitation was performed as described by Méchin *et al.* (2007), from the leaves of N⁺ and N⁻ plants harvested at the V and M stage of development. The frozen leaf powder

was resuspended in acetone with 0.07% (v/v) 2-mercaptoethanol and 10% (w/v) trichloroacetic acid. Proteins were allowed to precipitate for 1 h at -20 °C. The pellet was then washed overnight with acetone containing 0.07% (v/v) 2-mercaptoethanol. The supernatant was discarded and the pellet dried under vacuum. Protein resolubilization was performed according to Méchin *et al.* (2007) using 60 µl mg⁻¹ of R2D2 buffer [5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, 20 mM dithiothreitol, 5 mM TRIS (2-carboxyethyl) phosphine hydrochloride, 0.75% carrier ampholytes]. After resolubilization, samples were centrifuged and the supernatant was transferred to an Eppendorf tube prior to protein quantification. The total protein content of each sample was evaluated using the 2-D Quant kit (Amersham Biosciences).

Two-dimensional electrophoresis, gel staining, and image analysis

Solubilized proteins (300 µg) were separated on a pH 4–7 immobilized pH gradient (IPG) strip (Amersham Biosciences) using a Protean isoelectrofocusing (IEF) cell (Bio-Rad), as follows. Active rehydration was performed at 20 °C for 13 h at 50 V; then the focusing itself was carried out. For improved sample entry, the voltage was increased step by step from 50 V to 10 000 V (0.5 h at 200 V, 0.5 h at 500 V, 1 h at 1000 V, then 10 000 V for a total of 84 000 Vh). After IEF, strips were equilibrated to improve protein transfer to the two-dimensional gel (2-D gel). The second separation was performed in an 11% SDS–polyacrylamide gel. Separation was carried out at 20 V for 1 h and subsequently at a maximum of 30 mA per gel, 120 V overnight, until the bromophenol blue front had reached the end of the gel. After SDS–PAGE, the gels were subsequently stained with colloidal Coomassie blue. Scanning was carried out at 300 dpi with a 16-bit greyscale pixel depth using an image scanner (Amersham Biosciences), and then gel images were analysed using the Progenesis and SameSpot softwares (Nonlinear Dynamics Ltd). The SAS package [procedure GLM for one-way analysis of variance (ANOVA)] was used to examine modifications of individual protein spot volumes. For each protein spot, the mean normalized volume was then computed separately at the V and M plant developmental stage. A protein spot was selected if its variation had a *P*-value <0.05.

Protein identification by LC-MS/MS

Spot digestion and liquid chromatography–tandem mass spectrometry (LC-MS/MS) were performed as described by Martin *et al.* (2006). In-gel digestion was performed with the Progest system (Genomic Solution). Gel pieces were washed twice by successive separate baths of 10% acetic acid, 40% ethanol, and acetonitrile (ACN). The pieces were then washed twice with successive baths of 25 mM NH₄CO₃ and ACN. Digestion was subsequently performed for 6 h at 37 °C with 125 ng of modified trypsin (Promega) dissolved in 20% methanol and 20 mM NH₄CO₃. The peptides were extracted successively with 2% trifluoroacetic acid (TFA) and 50% ACN, and then with ACN. Peptide extracts were dried in a vacuum centrifuge and suspended in 20 ml of 0.05% TFA, 0.05% formic acid, and 2% ACN. HPLC was performed on an Ultimate LC system combined with a Famos Autosampler and a Switchos II microcolumn switch system (Dionex). Trypsin digestion was declared with one possible cleavage. Cysteine carboxyamidomethylation and methionine oxidation were set to static and variable modifications, respectively. A multiple-threshold filter was applied at the peptide level: Xcorr magnitude were up to 1.7, 2.2, 3.3, and 4.3 for peptides with one, two, three, and four isotopic charges, respectively; peptide probability <0.05, $\Delta Cn >0.1$ with a minimum of two different peptides for an identified protein. A database search was performed with Bioworks 3.3.1 (Thermo Electron). The TIGR maize gene indice database v 16, 72047*6 EST sequences (<http://compbio.dfci.harvard.edu/tgi/plant.html>) was used.

Metabolite extraction and analyses

Lyophilized leaf material was used for metabolite extraction. Approximately 20 mg of the powder was extracted in 1 ml of 80%

ethanol/20% distilled water for 1 h at 4 °C. During extraction, the samples were continuously agitated and then centrifuged for 5 min at 15 000 rpm. The supernatant was removed and the pellet was subjected to a further extraction in 60% ethanol and finally in water at 4 °C, as described above. All supernatants were combined to form the aqueous alcoholic extract. Nitrate was determined by the method of Cataldo *et al.* (1975). Total free amino acids were determined by the Rosen colorimetric method with leucine as a standard (Rosen, 1957). Chlorophyll was estimated using 10 mg of fresh leaf material (Arnon, 1949). The total N content of 2 mg of lyophilized material was determined in a N elemental analyser using the combustion method of Dumas (Flash 2000, Thermo Scientific, Cergy-Pontoise, France).

Metabolome analysis

All steps were adapted from the original protocol described by Fiehn (2006). All extraction steps were performed in 2 ml of Safelock Eppendorf tubes. The ground frozen leaf samples were resuspended in 1 ml of frozen (−20 °C) water:chloroform:methanol (1:1:2.5) and extracted for 10 min at 4 °C with shaking at 1400 rpm in an Eppendorf Thermomixer. Insoluble material was removed by centrifugation and 900 µl of the supernatant were mixed with 20 µl of 200 µg ml^{−1} ribitol in methanol. Water (360 µl) was then added and, after mixing and centrifugation, 50 µl of the upper polar phase were collected and dried for 3 h in a Speed-Vac and stored at −80 °C. Four blank tubes were subjected to the same steps as the samples.

For derivatization, samples were removed from −80 °C storage, warmed for 15 min before opening, and Speed-Vac dried for 1 h before the addition of 10 µl of 20 mg ml^{−1} methoxyamine in pyridine. The reactions with the individual samples, blanks, and amino acid standards were performed for 90 min at 28 °C with continuous shaking in an Eppendorf thermomixer. A 90 µl aliquot of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was then added and the reaction continued for 30 min at 37 °C. After cooling, 50 µl of the reaction mixture were transferred to an Agilent vial for injection.

For the analysis, 3 h and 20 min after derivatization, 1 µl of the derivatized samples were injected in the Splitless mode onto an Agilent 7890A gas chromatograph (GC) coupled to an Agilent 5975C mass spectrometer (MS). The column used was an Rxi-5SilMS from Restek (30 m with 10 m Integra-Guard column). The liner (Restek # 20994) was changed before each series of analyses and 10 cm of the column were removed. The oven temperature ramp was 70 °C for 7 min, then 10 °C min^{−1} up to 325 °C, which was maintained for 4 min. Overall, the total run time was 36.5 min. A constant flow of helium was maintained at 1.5231 ml min^{−1}. Temperatures in the GC were the following: injector, 250 °C;

transfer line, 290 °C; source, 250 °C; and quadrupole, 150 °C. Samples and blanks were randomized. Amino acid standards were injected at the beginning and end of the analyses for monitoring of the derivatization stability. An alkane mix (C10, C12, C15, C19, C22, C28, C32, and C36) was injected in the middle of the analyses for external retention index calibration. Five scans per second were acquired. For data processing, Raw Agilent data files were converted into the NetCDF format and analysed with AMDIS <http://chemdata.nist.gov/mass-spc/amdis/>. A home retention indices/mass spectra library built from the NIST, Golm, and Fiehn databases and standard compounds was used for metabolite identification. Peak areas were then determined using the quanlynx software (Waters) after conversion of the NetCDF file into the masslynx format. Statistical analyses were carried out with TMEV <http://www.tm4.org/mev.html>. Univariate analyses by permutation (one- and two-way ANOVA) were first used to select the metabolites exhibiting significant changes in their concentration.

Results and Discussion

Impact of long-term N starvation on plant N status, plant phenotype, and yield

To determine the impact of N deficiency on plant N status, phenotype, and kernel production, plants were grown in a glasshouse until maturity under low (N[−]) and high N (N⁺) fertilization conditions. At the V and the M developmental stages, plants exhibited a similar pattern of development, including the flowering time. Although the N[−] plants were chlorotic at both the V and M stages, they were a similar height and had approximately the same number of leaves (Fig. 1). In contrast, a major decrease in yield mainly due to a reduction in kernel number was observed in N[−] plants at the time of harvest (Fig. 1). The values for yield and its components were in N⁺, 117±9 g for kernel yield (KY), 464±21 for kernel number (KN), and 252±17 g for thousand kernel weight (TKW); and in N[−], 15±4 g for KY, 55±10 for KN, and 270±17 g for TKW. Such a result confirms that N deficiency causes a strong yield penalty (Ribaut *et al.*, 2007), but has a limited effect on plant phenotype in terms of leaf number and overall plant development (Dubois *et al.*, 2003). The effect of N

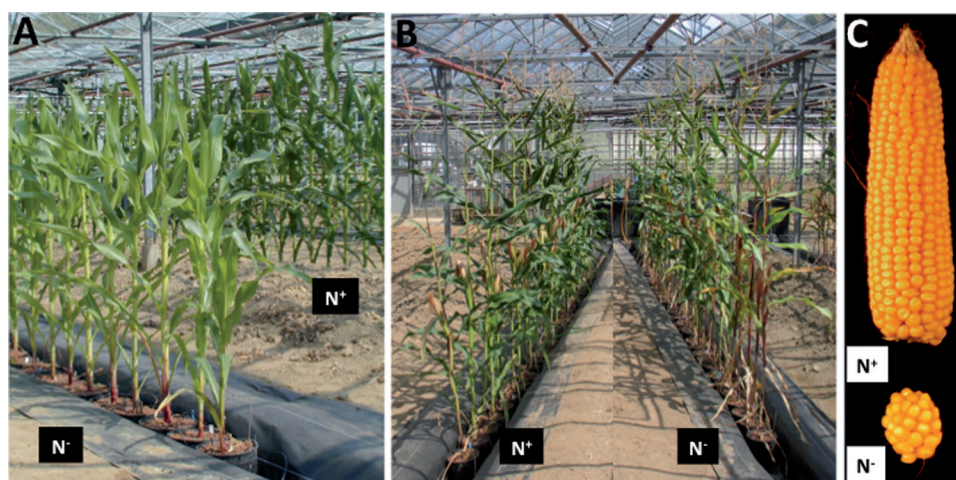


Fig. 1. Shoot and ear phenotype of maize plants grown under low and high N fertilization conditions. (A) Plants at the V stage grown under low (N[−]) and high N supply (N⁺). (B) Plants at the M stage. (C) Phenotype of the ear. The plants were grown in the glasshouse on clay loam soil and watered daily with the low and high N nutrient solutions as described in the Materials and methods.

deficiency on representative markers of leaf N status was examined at three key stages of plant development: the V stage, the F stage, and the M stage (Table 1). Except for the NO_3^- content, there was a 2- to 4-fold decrease in the amount of most of the marker traits representative of the plant N status, such as chlorophyll, total N, amino acids, and protein, in the N^- plants. The leaf NO_3^- content remained high at both the F and the M stages of plant development, whichever the N fertilization regime, due to the ability of maize to still absorb considerable amounts of mineral N after flowering (Hirel *et al.*, 2007). When considering the differences in most of the N marker traits of the leaves for each of the two N fertilization regimes, the strongest and most significant decrease was observed between the V and the M stage, which prompted the selection of these two developmental stages for further 'omics' analysis. Moreover, the M developmental stage was chosen because the changes in a number of physiological and molecular biomarkers representative of N remobilization indicate that in the leaf below the ear, the transition from sink to source takes place in this period (Hirel *et al.*, 2005; Martin *et al.*, 2005, 2006).

Long-term N starvation induces drastic changes in the leaf metabolite profile at the vegetative stage

In the present study, GC/MS analysis of the leaf metabolome was performed using plants grown at low (N^-) and high (N^+) N supply at two key stages of plant development: the V stage and 55 DAS, referred to as leaf maturity (M). At the V stage, during which young developing leaves efficiently assimilate C through the photosynthetic process and inorganic N for amino acid and protein synthesis (Masclaux *et al.*, 2001), extensive differences were observed in metabolite accumulation under N-deficient conditions (Supplementary Table S1 available at *JXB* online). Examples of the differences observed in the accumulation of a number of C and N metabolites involved in the main enzymatic reactions occurring in a leaf of a C_4 plant at the V stage under the N^- and N^+ conditions are presented in Fig. 2. Among the 71 identified metabolites that showed statistically significant differences in the three replicates ($P \leq 0.05$), the concentration of most of the amino acids (18 out of 22), as well as a number of N-containing molecules derived from glutamine and glutamate, such as γ -aminobutyric acid (GABA), was strongly reduced from 4- to 37-fold under N^- conditions. In addition, a significant

decrease was observed in the amount of a number of organic acids involved in the tricarboxylic acid (TCA) cycle and in C_3 and C_4 carbon metabolism, notably phosphoenolpyruvate (PEP) and malate, and of soluble carbohydrates such as glucose and fructose. Under N^- conditions, the glucose 6-P and fructose 6-P contents were also reduced, indicating that there was a lower accumulation of some intermediates of the glycolytic pathway or of sucrose and starch biosynthesis. In addition, sedoheptulose was detected in lower concentrations, in line with previous findings showing that at optimal N nutrition, the photosynthetic capacity of mature leaves in maize is increased (Huber *et al.*, 1989).

Concerning other reactions of N metabolism, the lower concentration of urea indicates that arginine catabolism in the urea cycle (Slocum, 2005) was slower in leaves of N-deficient plants. Lower amounts of allantoin were also detected, a molecule that may be used for N storage, N translocation, or N signalling (Reinbothe and Mothes, 1962; Witte, 2011). Interestingly, a reduction in the concentration of several unusual and minor metabolites was also observed. These were mostly represented by organic acids other than those involved in the TCA cycle, such as glucarate, tartrate, glutarate, and 2-hydroxyglutarate (Ornston, 1977). The finding that the arabinose and galactose contents were reduced in the N^- plants suggests that plant cell wall biosynthesis was modified (Burget *et al.*, 2003; Linster and Clarke, 2008). A decrease in lactose, mannose, pentitol, and sorbitol was also detected, that may have different metabolic and signalling functions (Pego *et al.*, 2000; Lerouxel *et al.*, 2006; Kanayama *et al.*, 2008). Differences in the concentrations of shikimate, phenylpropanoids such as quinate, coumaroylquininate, caffeoylquininate, and feruloylquininate, and closely related compounds such as salicylate and sinapinate (Bernards, 2010) and of a number of unknown molecules similar to chlorogenates [referred to as (U) chlorogenates; Supplementary Table S1 at *JXB* online] suggest that, under N deficiency, lignin biosynthesis was altered (Fritz *et al.*, 2006). Among the rest of the unusual compounds found in lower concentrations under N-deficient conditions was pimelate, a molecule originating from fatty acids and used for biotin synthesis (Webb *et al.*, 2007), and erythritol, a precursor of natural compounds such as carotenoids (Cordoba *et al.*, 2009).

In comparison with those metabolites exhibiting a decrease in concentration, there were far fewer metabolites exhibiting a higher level of accumulation under N-deficient conditions. As

Table 1. Concentration of nitrogen metabolites in maize leaves at three development stages when plants were grown under low and high N fertilization conditions

Metabolites were quantified in maize leaves at the vegetative stage (V), 15 days after silking (DAS; F), and 55 DAS (M). The concentration of N metabolites is expressed in $\mu\text{mol g dry weight (DW)}^{-1}$ and total N as %. Chlorophyll concentration is expressed as mg g DW^{-1} .

	N^+			N^-		
	V	F	M	V	F	M
Total N	4.36±0.08	3.03±0.18	2.42±0.16	1.80±0.15**	1.12±0.18**	0.6±0.16*
NO_3^-	33.7±1.07	15.3±0.05	15.2±0.05	6.3±0.06*	14.7±0.16	15.7±0.32
Amino acids	121.11±6.23	163.02±31.02	150.08±19.01	58.36±16.3*	31.58±0.15*	34.42±13.11*
Proteins	132.59±0.71	84.24±7.02	58.6±3.63	58.2±2.82**	26.24±1.19**	22.96±0.26**
Chlorophyll	1.87±0.16	3.61±0.43	2.51±0.11	0.69±0.06*	1.24±0.19*	0.22±0.02***

Values are the mean of three replicates ±SE. For each of the three plant developmental stages *, **, *** indicate significant differences at the 0.05, 0.01, and 0.001 probability level, respectively, between the two levels of N fertilization, high (N^+) or low (N^-).

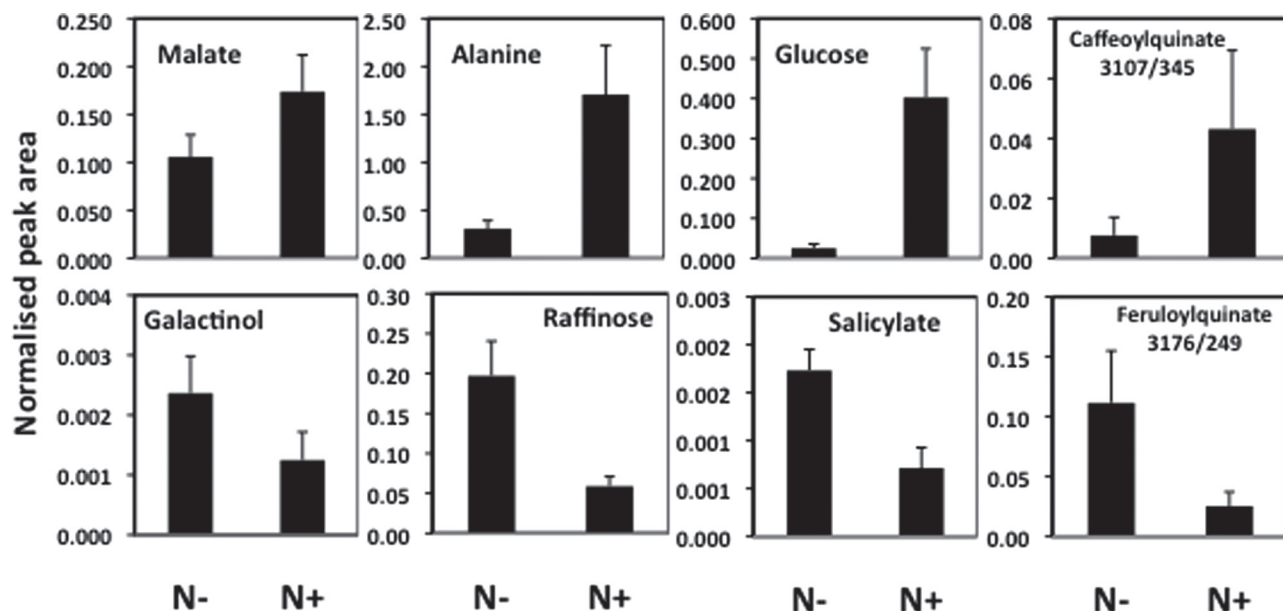


Fig. 2. Representative maize leaf metabolites exhibiting changes in their concentration, extracted from plants grown under low and high N fertilization conditions. Metabolite concentrations were measured in leaves at the vegetative stage (V) in maize plants grown under high (N⁺) or low (N⁻) nitrogen supply. Leaf metabolomic analysis is presented in Supplementary Table S1 at *JXB* online, and was performed as described in the Materials and methods. For these metabolites, significant differences between N⁺ and N⁻ were observed at $P < 0.05$ using the Student *t*-test. The numbers for caffeoylquininate and feruloylquininate refer to the compound identification number in the metabolome analysis.

already observed in maize and other species, the amount of starch was much higher in the leaves of plants grown under low N input (Hirel *et al.*, 2005; Terce-Laforgue *et al.*, 2004). This occurs when the plant reduces the production of organic acids and a number of C molecules derived from photosynthesis that are not required to synthesize amino acids (Sheible *et al.*, 1997). The increase in the concentration of galactinol, raffinose, maltose, mannitol, trehalose, and rhamnose previously observed in N-deficient *Arabidopsis* plants (Krapp *et al.*, 2011) suggests that the metabolic response caused by prolonged N deficiency is similar to that found for many other abiotic stresses (Stoop *et al.*, 1996) such as high and low temperature stress (Kaplan and Guy, 2004). Their increase probably occurs partly at the expense of the precursor molecules fructose and glucose, since they were detected at a lower concentration in the N⁻ plants. The snapshot provided by the metabolic profile at the V stage makes available an integrated picture of the leaf metabolic status under N-deficient conditions (Fig. 3).

In conclusion, these metabolomic results suggest that N availability is probably the main driver controlling both primary and secondary leaf C metabolism during vegetative development. To determine whether this process occurs at a later stage of plant development, the same study was conducted on leaves of plants at the M stage.

The leaf metabolic profile at maturity is less altered by long-term N deficiency

At the M stage during the kernel-filling period, leaves efficiently remobilize N and export C for kernel filling. At this stage of plant development, the metabolic profile of the leaf below the ear was very different compared with that of a leaf of a plant at the V stage, and far fewer metabolites (44 instead of 71) exhibited differences

in their concentration under N-deficient conditions (Supplementary Table S2 at *JXB* online). There was still a reduction in the amount of several organic acids in the TCA cycle such as pyruvate and aconitate and those derived from the TCA cycle such as galactarate. At the M stage, N deficiency leads to a decrease in the amounts of several soluble carbohydrates, galactolipids, and sugar alcohols. This decrease was observed for those usually present in plants such as sucrose, mannose, and galactose, but also for melibiose, gentiobiose, and galactosylglycerol for which the biosynthesis and physiological roles are not clearly defined. A reduction in the concentration of the three polyamines spermidine, tyramine, and putrescine and also of the catecholamine dopamine suggests that under N deficiency, the synthesis of a number of compounds involved in a variety of developmental and signalling processes (Altman and Levin, 1993; Culma and Szopa, 2007) decreases during the M phase. Interestingly, the decrease in the content of most of the amino acids observed at the V stage did not occur at the M stage. Under N-deficient conditions at the M stage, only tyrosine, threonine, alanine, leucine, and methionine were present in lower concentrations (2- to 5-fold lower). It is likely that their synthesis still occurs at the M stage when N is not limiting. Thus, it will be interesting to determine whether, for example, the decrease in alanine is related to the importance of the enzyme alanine aminotransferase in the improvement of plant productivity in general and NUE in particular (Good and Beatty, 2011).

The leaf metabolic profile is specific to the plant developmental stage

If the differences in the metabolite concentrations that are common to the two plant developmental stages (V and M) are

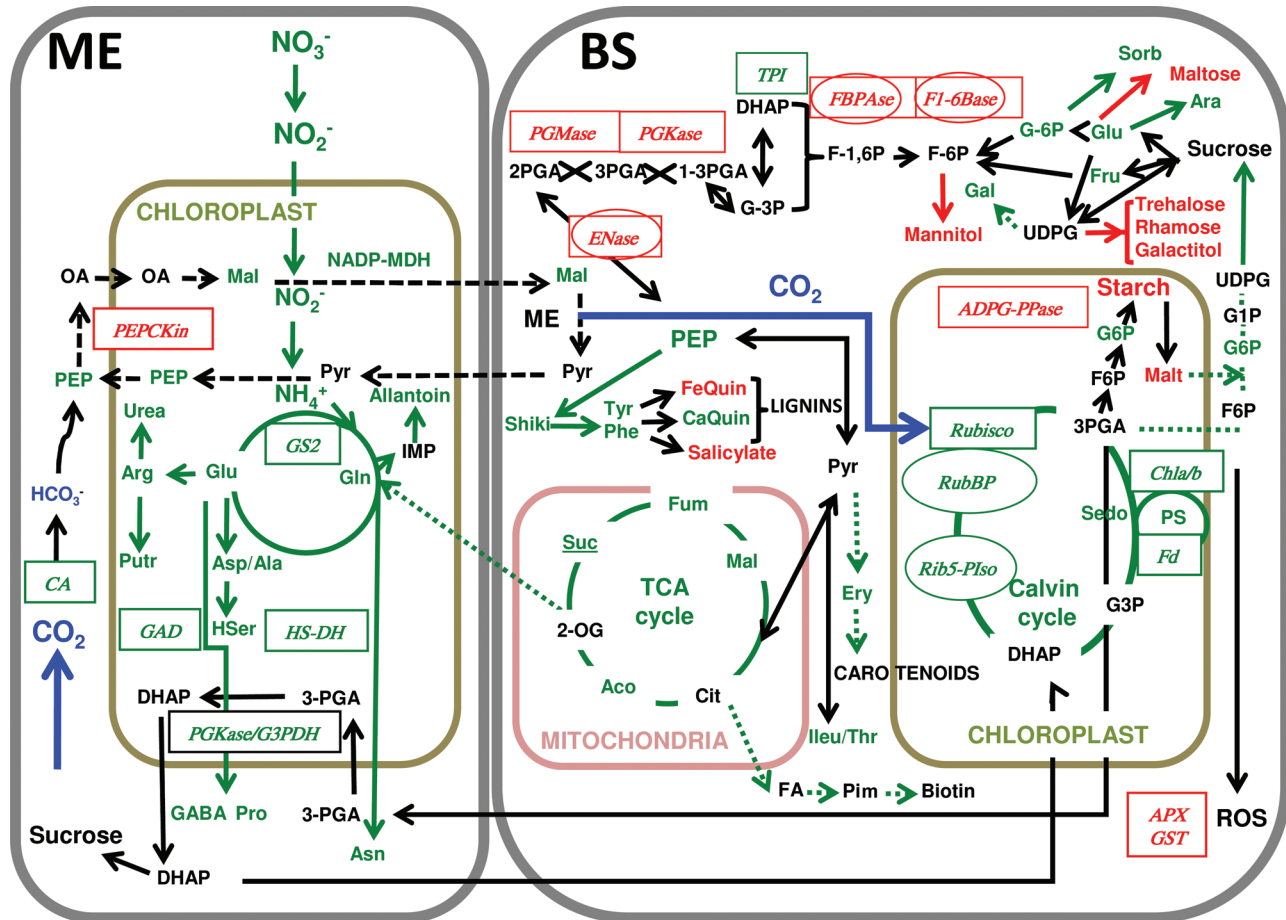


Fig. 3. Schematic representation of the physiological changes occurring in a young developing maize leaf when N is limiting. A simplified representation of the main metabolic pathways involved in C and N metabolism and their distribution between the mesophyll (ME) and bundle sheath (BS) cells is shown. Metabolomic analysis was performed on a leaf at the vegetative (V) stage. Metabolites detected at lower or higher concentrations in N^- are in green characters and red characters, respectively. The main changes observed in the amounts of proteins and transcripts for proteins involved in the corresponding biochemical pathways are also indicated in green (lower amount) or red (higher amount) with a circle for the proteins and a square for the transcripts. Abbreviations for proteins: ENase, enolase; PGMase, phosphoglucomutase; PGKase, phosphoglucokinase; FBPase, fructose bisphosphatase; F1-6Base, fructose 1,6 bisphosphatase; RubBP, Rubisco-binding protein; Ri5-Iso, ribose-5-phosphate isomerase; Chla/b, chlorophyll *a/b* complex. Abbreviations for transcripts: PEPCKin, phosphoenolpyruvate carboxylate kinase; ADPG-PPase, ADP-glucose pyrophosphorylase; APX, ascorbate peroxidase; GST, glutathione-S-transferase; CA, carbonic anhydrase; GS2, plastidic glutamine synthase; GAD, glutamate decarboxylase; HSDH, homoserine dehydrogenase; PGKase, phosphoglucokinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Rubisco, ribulose1-5-bisphosphate carboxylase; Fd, ferredoxin; TPI, triose phosphate isomerase. The green and red lines correspond to putative down-regulation or up-regulation of a metabolic pathway, respectively, when N is limiting.

examined, only a limited number of metabolites are concerned. For those detected in lower concentrations under N deficiency, there are a number of amino acids such as alanine, tyrosine, lysine, threonine, and methionine, and various C-containing molecules such as erythronate, glycerol-P, galactose, mannose, and glucopyranose. Urea and putrescine were also present in lower amounts at both the V and M stage. Interestingly, no metabolite that exhibited a decrease in concentration at both the V and M stage was found. In contrast, for others, there was apparently a metabolic adjustment that occurred between the V and the M stage, since divergent patterns of accumulation were observed for a number of metabolites. For example, under N deficiency, there was less fumarate and erythritol at the V stage, whereas they were detected at higher concentrations at the M stage. In

contrast, pipercolate was present at a higher concentration at the V stage, but was detected at a lower concentration at the M stage.

The leaf proteome under long-term N deficiency is not markedly modified on a functional basis

At the V stage, when the proteomes of N^- and N^+ leaves were compared, it was observed that the volume of 45 protein spots exhibited significant differences ($P < 0.05$). Only 40 proteins were identified. The densitometric quantification of these differences is presented in Supplementary Table S3 at JXB online. At the M stage, the volumes of 40 protein spots were significantly modified in the N-deficient leaves; however, only 34 proteins were identified (Supplementary Table S4). An example of the

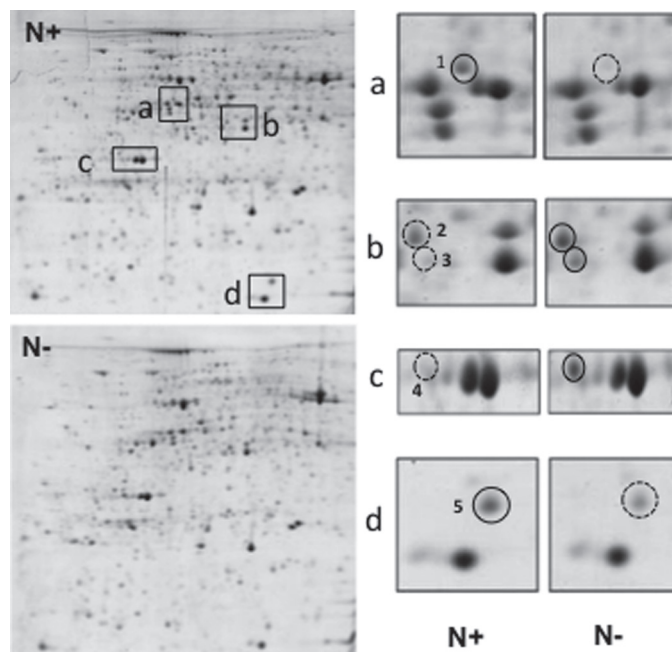


Fig. 4. Proteomic analysis of maize leaves from plants grown under low and high N fertilization conditions. Left: example of 2-D gels of leaf proteins at the vegetative (V) stage of maize plants grown under high (N^+) or low (N^-) nitrogen supply. The squares a, b, c, and d correspond to representative regions of the gel shown on the right side of the panel, in which there were quantitative differences in the intensities of the leaf protein spots. Spot 1, δ -aminolaevulinic acid dehydratase, chloroplast precursor {Hv}; spot 2, fructose 1,6-bisphosphatase, cytosolic {Sh}; spot 3, fructose bisphosphatase aldolase class-I {Os}; spot 4, oxygen-evolving enhancer protein 1 chloroplast precursor {Ta}; spot 5, glycine-rich protein {Zm}. The quantification of the proteins spots is presented in Supplementary Table S3 at *JXB* online and was performed as described in the Materials and methods. The circles with full lines show protein spots present at higher concentration and the circles with dotted lines show proteins that are present at lower concentration either in N^+ or in N^- . For these proteins, significant differences between N^+ and N^- were observed at $P < 0.05$ using the Student *t*-test.

modifications occurring in the spectrum of leaf proteins, showing the differences between the N^+ and N^- plants at the V stage, is shown in Fig. 4.

At the V stage, among the 40 identified proteins, it was observed that 26 were present at a lower concentration when N was limiting. These were involved in a variety of physiological processes including C assimilation, protein stabilization, protein degradation, oxidative stress, plant defence mechanisms, the cell wall, and lignin metabolism. Concerning the C assimilatory pathway, it was observed that under N-deficient conditions, there was a lower amount of the ribose-5-phosphate isomerase precursor. This precursor would eventually produce a functional enzyme of the Calvin cycle (Martin *et al.*, 1996). This result indicates that N assimilation and some of the processes ensuring efficient C assimilation are coordinately regulated in terms of protein accumulation. Other processes not directly linked to leaf photosynthetic activity, such as those involved in oxidative stress, also appear to be down-regulated at the protein level. For example, there was a lower amount of peroxiredoxin, an antioxidant enzyme that protects chloroplastic membranes against photooxidative damage (Baier *et al.*, 1999; Dietz, 2011). Although limited to a reduction in the amount of two proteins involved in cell wall synthesis (glycine-rich protein) and lignin biosynthesis (cinnamyl alcohol dehydrogenase), it can be concluded that N deficiency probably alters some of the biological processes involved in plant growth and development.

Among the relatively low number of proteins that were present in a higher quantity in leaves at the V stage when plants were grown under N-deficient conditions, it was interesting to observe that four of them corresponded to enzymes involved in the glycolytic pathway. An increase in the level of glycolytic enzymes such as enolase, fructose-bisphosphatase, aldolase, and fructose 1,6-bisphosphatase associated with an increase in respiration is generally observed when there is a decrease in photosynthesis (Stitt *et al.*, 1990). This increase is apparently related to the regulation of the sink capacity of the plant that is strongly reduced, particularly when N is limiting. The function of the two proteins, fibrillin and hairpin-binding protein, which may be involved in the plant response to various stresses, is still not fully understood (Yang *et al.*, 2008; Singh and McNellis, 2011). Strikingly, in rice, these two proteins have been identified previously as being N deficiency stress-responsive proteins (Song *et al.*, 2011).

At the M stage, the differences in protein profiles of the N^- and N^+ leaves were not the same as those detected at the V stage. Although limited to a relatively small number of proteins, the differences indicate that the main metabolic functions involved in providing C and N assimilates to the kernels through photosynthesis, C assimilation, and protein remobilization were considerably altered. Strikingly, for a number of these proteins, such as those involved in the formation of chlorophyll–protein complexes, N deficiency had an opposite effect, since some of them were present in higher amounts and others in lower amounts when

N was limiting. It is likely that these opposite effects are related to the capacity of the plant to react and to respond differently to a stress by adjusting the expression of members of a multigene family or of a set of genes involved in key biological functions (Chavese *et al.*, 2009). The complex and differential effect of N deficiency on protein accumulation is also illustrated by the finding that arginine decarboxylase and *S*-adenosylmethionine synthetase, two key enzymes involved in polyamine biosynthesis (Kumar *et al.*, 1997), were present in lower and higher amounts, respectively, when N was limiting. Interestingly, the amount of cysteine synthase was lower under N deficiency, suggesting that other metabolic processes such as sulphur metabolism (Witz and Hell, 2006) were altered at this particular stage of plant development. Moreover, there is plenty of evidence of a strong relationship between sulphur and N metabolism (Hell, 1997).

In conclusion, it is somehow surprising to find that relatively few proteins exhibited significant differences in their level of accumulation following long-term N deficiency, irrespective of the plant developmental stage. This is partly due to the fact that proteomic techniques in maize allow only the detection of a limited number of proteins (Porubleva *et al.*, 2001), even under long-term stress conditions (Li *et al.*, 2011). This may also be because there were apparently very few differences in the proteins identified in N⁻ and N⁺ leaves in comparison with those found in the spectrum of both metabolites (see above) and transcripts (see below). There also needs to be taken into consideration the fact that under N-deficient conditions, at both the V and the M stage of plant development, the amount of total soluble protein per unit of leaf dry weight was reduced by over half (Table 1). This will probably mean that all proteins are reduced in the leaves that have been subjected to N deficiency and that the data obtained by proteomic analysis discussed above refer to relative differences, as equal amounts of protein were analysed. At this stage of the investigation, the proteomic approach did not consider the whole protein profile and the post-translational modifications known to be of major importance in response to plant biotic and abiotic stresses. Among these post-translational modifications, there is phosphorylation that alters protein charge and occurs in a number of signalling mechanisms (Kosova *et al.*, 2011). Protein oxidation (Gill *et al.*, 2010) and lysine acetylation have also been shown to be involved in the response to environmental cues including the regulation of both primary C and N metabolism (Finkemeier *et al.*, 2011).

Nevertheless, in agreement with a previous study, it is clear that N deficiency stress alters mostly the accumulation of enzymes involved in primary C metabolism and closely related metabolic pathways (Bahrman *et al.*, 2004). Interestingly, the alterations of the protein profile shared several common characteristics with those occurring after viral infection, such as the up-regulation of enzymes involved in starch biosynthesis or glycolysis (Li *et al.*, 2011). Differences in the protein profile of maize (Prinsi *et al.*, 2009) and rice (Kim *et al.*, 2009; Song *et al.*, 2011) have recently been investigated, when plants were grown under low and high fertilization conditions. These two studies showed that the amounts of enzyme proteins having a pivotal role in N assimilation, such as glutamine synthase (GS), and in C metabolism, such as PEP carboxylase, were higher when plants were fed with nitrate. Many other proteins involved in a number of

photosynthetic reactions and in maintaining the energy and the redox status of the cell were also shown to be N responsive. Both of these previous studies and the present investigation confirm that there is a tight relationship between C and N metabolism and their energy requirement, not only at the transcriptional and metabolic level (Nunes-Nesi *et al.*, 2010; Krapp *et al.*, 2011), but also at the proteomic level.

Correlations between N deficiency, mRNA accumulation, and a variety of metabolic, signalling, and developmental functions

Both at the V and at the M plant developmental stage, the amount of leaf mRNA transcripts following long-term N deficiency was studied using a maize whole genome oligonucleotide microarray. The corresponding genes were classified into different functional categories defined according to both their names and their putative annotations.

At the V stage, it was observed that 74 transcripts were present in lower amounts in N⁻ leaves (Supplementary Table S5a at *JXB* online). The biological processes that were the most significantly reduced in N⁻ leaves were photosynthesis, C assimilation, and C metabolism (grouped in the same class referred to as C metabolism in Fig. 5), followed by various other metabolic processes involved, for example, in metal homeostasis/detoxification (transcripts for various metallothioneins), plant pigment synthesis (transcripts for indole synthase), and phospholipid biosynthesis (transcripts for phosphoethanolamine *N*-methyltransferase). The reduction in the transcripts suggests that the regulation of a variety of other biological processes was modified, such as those involved in various transport mechanisms, proteolysis, signalling, and glycolysis, the latter being included in C metabolism in Fig. 5. Surprisingly, in the N⁻ leaves, there were few differences in the genes encoding functions related to primary N metabolism. Only the transcripts for plastidic GS (GS2) and for homoserine dehydrogenase (an enzyme involved in the synthesis of threonine, isoleucine, and methionine) were detected in lower amounts. These results contrast with those of a number of reports in which the authors found that both in maize (Chen *et al.*, 2011; Yang *et al.*, 2011) and in *Arabidopsis* (Bi *et al.*, 2007; Krapp *et al.*, 2011), the expression of a considerable number of genes encoding enzymes or proteins involved in N metabolism was down-regulated. These differences in terms of gene expression patterns may be due to either the intensity or the duration of the N deficiency stress. The developmental stage of the plant and the species, or for maize the genotype, may also explain the differences observed by the different groups (Cañas *et al.*, 2010). The two other classes of transcript that were down-regulated under N-deficient conditions were involved in the translation machinery (cytosol and chloroplast) and various transport systems including sugars and proteins.

It was found that at the V stage, the transcripts for 76 genes were present in higher amounts in the N⁻ as compared with N⁺ leaves (Supplementary Table S5b at *JXB* online). Transcripts for various genes involved in different signalling functions were the most abundantly represented, followed by those encoding proteins involved in various stresses, transport mechanisms, and cell wall synthesis (Fig. 5). For a number of genes, leaf transcriptional activity was presumably also up-regulated,

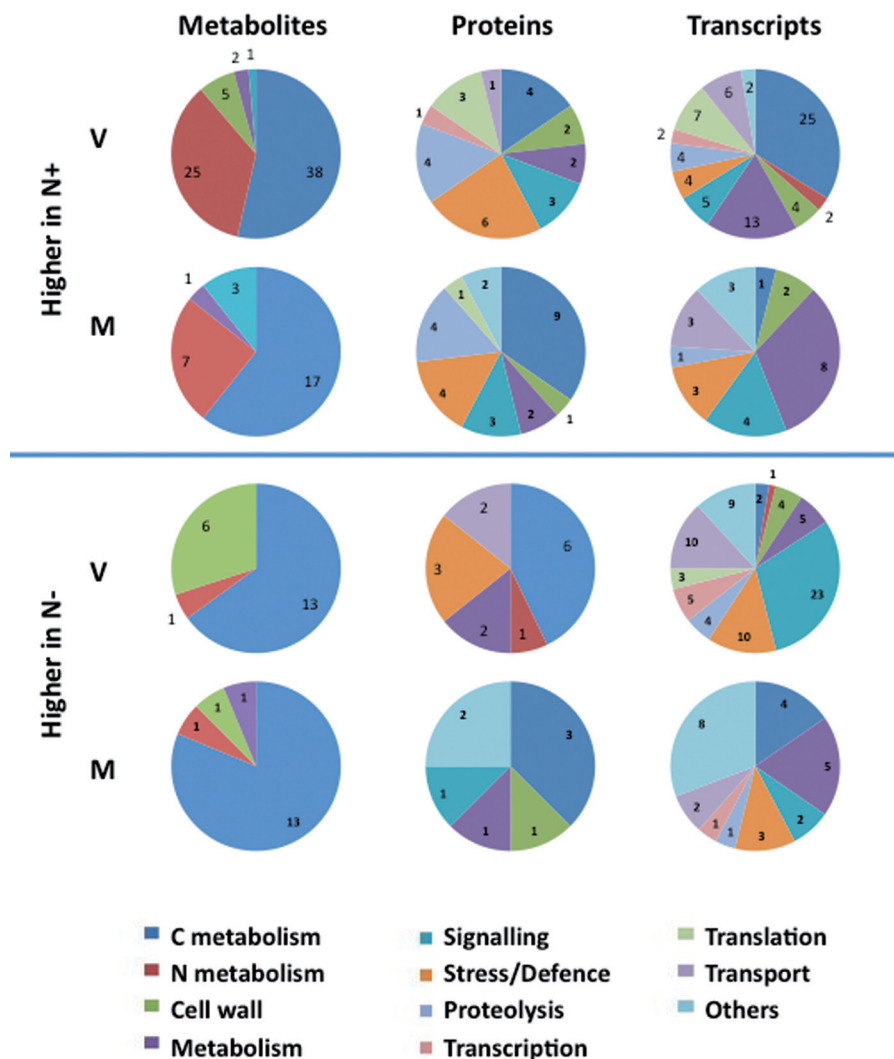


Fig. 5. Functional categories of metabolites, proteins, and gene transcripts isolated from leaves of maize plants grown under low and high N fertilization conditions, exhibiting differences in their level of accumulation. Pie charts show the number of metabolites, proteins, and transcripts identified in the three ‘omics’ experiments exhibiting an increase under high (N⁺) or low (N⁻) nitrogen supply at the vegetative (V) and mature (M) stage of leaf development.

since higher amounts of transcripts encoding DNA-binding proteins were detected. Several studies have shown that a number of genes involved in a variety of metabolic and signalling processes are differentially expressed following a mild or a severe chronic N stress. These studies, mostly performed on the model plant *Arabidopsis*, have highlighted the fact that there is a complex network of responses to N deficiency (Lian *et al.*, 2006; Bi *et al.*, 2007; Lopes and Araus, 2008; Vidal *et al.*, 2010; Chen *et al.*, 2011). However, it appears that only a small subset of transcripts share a common pattern of accumulation with those of previously published studies. This is probably due to different experimental conditions and because of species specificities in terms of N management at both the physiological and molecular levels. Nevertheless, these studies have highlighted the fact that there is a complex interaction between N signalling and a number of other metabolic pathways and developmental processes (Walch-Liu *et al.*, 2005; Nunes-Nesi *et al.*, 2010; Vidal *et al.*, 2010). These interactions were revealed by the differences observed in the accumulation of transcripts for genes encoding a vast repertoire of proteins

involved in signal transduction mechanisms (Wang *et al.*, 2003; Yang *et al.*, 2011). For comparison, a list of maize genes found to be N responsive both in the work of Yang *et al.* (2011) and in the present study is presented in Table 2. It is, however, worth stressing that among the marker genes identified in the two studies, a number of them exhibited an opposite pattern of mRNA accumulation, probably due either to the genetic background or to different experimental conditions. It is also clear that the plant developmental stage appears to be of importance, since a number of the N-responsive genes identified in the present study were also found to be up- or down-regulated during leaf senescence (Gregersen and Bach Holm, 2006). During the onset of leaf senescence, N remobilization is triggered almost simultaneously because the plant reduces N uptake and utilization, thus mimicking N-deficient conditions (Masclaux *et al.*, 2000; Tercé-Laforgue *et al.*, 2005).

Although it is difficult to describe in detail the function of each individual N-responsive gene, they have been classified into specific functional categories that provide an overview of the corresponding transcript accumulation pattern at the

Table 2. List of genes showing changes in the level of the corresponding transcripts under low and high N nutrition regimes in two different studies

The genes were selected from the present study and that of Yang *et al.* (2011) using their functional annotations.

Study of Yang <i>et al.</i> (2011)			Present study					
Probe set	SmartBlast annotation	UniRefID	Expression pattern	Stage	Expression pattern	Gene index	Functional category	Putative annotation
A1ZM019124_at	Putative acid phosphatase: 3e-77	Q9M4D6	Higher in N ⁺	V	Higher in N ⁺	TC252728	Signalling	Putative acid phosphatase {Hv} CAB71336.2
A1ZM019124_s_at	Putative acid phosphatase: 3e-77	Q9M4D6	Higher in N ⁺	V				
A1ZM055541_at	Phosphoenolpyruvate carboxylase kinase 4: 1e-166	Q5EC56	Higher in N ⁺	V	Higher in N ⁻	TC264264	C metabolism	Phosphoenolpyruvate carboxylase kinase {Sb} Q5EC56
A1ZM001405_at	Pathogenesis-related protein-5: 7e-99	O82087	Higher in N ⁺	V	Higher in N ⁻	TC274865	Defence	Pathogenesis-related protein-1 {Zm} AAC25629.1
A1ZM041999_s_at	ADP-glucose pyrophosphorylase small subunit: 0.0	Q941P2	Higher in N ⁺	V	Higher in N ⁻	TC251067	C metabolism	ADP-glucose pyrophosphorylase small subunit {Zm}
A1ZM084469_at	Cellulose synthase catalytic subunit-like protein: 2e-39	Q9LFL0	Higher in N ⁻	V	Higher in N ⁻	TC255800	Cell wall	Putative cellulose synthase-like protein OsCsIE2 {Os}
A1ZM004697_at	E-class P450, group I: 0.0	Q2HV82	Higher in N ⁻	V + M	V, higher in N ⁻ ; M, higher in N ⁺	TC278653	Metabolism	Putative cytochrome P450 71C4 {Os} BAD08938.1
				V	Higher in N ⁺	TC270588	Metabolism	Cytochrome P450 71C2 {Zm} T03034
A1ZM021366_s_at	Salt-stress root protein RS1: 3e-49	P83649	Higher in N ⁻	V	Higher in N ⁻	TC259486	Stress	Salt-stress root protein RS1 {Os} BAA96588.1
A1ZM015924_at				V	Higher in N ⁻	TC259487	Stress	Salt-stress root protein RS1 {Os} BAA96588.2
A1ZM010913_at	Proline-rich protein family-like: 6e-43	Q6Z1G1	Higher in N ⁺	V	Higher in N ⁺	TC261242	Cell wall	Proline-rich protein {Zm} CAB91647.1
A1ZM060059_at	Proline-rich region: 1e-08	Q0IBI7	Higher in N ⁻	V				
A1ZM022541_a_at	Myb family transcription factor-like: 7e-21	Q6Z869	Higher in N ⁺	V	Higher in N ⁻	TC264482	Signalling	Putative transcription factor (Myb-like DNA-binding domain) {Os} PF00249
A1ZM056023_x_at	Putative MYB transcription factor: 2e-41	Q5N9B7	Higher in N ⁻	V	Higher in N ⁻	AZM4_1726	Transcription	Myb factor protein {Os} BAB63829.1
				V	Higher in N ⁺	TC263292	Transcription	Putative Myb-like DNA-binding protein {Os}
A1ZM055587_x_at	Putative endo-1,3-beta-glucanase: 1e-137	Q8S9Q6	Higher in N ⁺	V	Higher in N ⁻	TC269346	Cell wall	Putative endo-1,3;1,4-beta-D-glucanase {Os}
				V	Higher in N ⁺	TC259998	Cell wall	Putative endo-1,4-beta-glucanase {Os} AAP03405.1

V stage. This overview indicates that at the transcript accumulation level, the expression of genes encoding proteins involved in photosynthesis, C metabolism, C assimilation, and a variety of metabolic or transcriptional processes was altered when N was limiting. Conversely, N deficiency induced a number of specific responses in terms of mRNA accumulation, involving those encoding transcription factors, stress-responsive elements, and proteins transporting various organic and mineral compounds (Figs. 3, 5).

At least in maize, no transcriptome study has been carried out that takes into account the plant developmental stage. If it is

considered that there are profound differences at both the physiological and molecular levels during the transition from N assimilation to N remobilization in a variety of plant species including maize (Masclaux *et al.*, 2000; Hirel *et al.*, 2005), this is a major omission. Surprisingly, under N-deficient conditions, no decrease or increase in the amount of transcripts common to both the V and M stage was found (Supplementary Table S6 at *JXB* online). This observation suggests that the regulation of gene expression is tightly dependent upon the plant developmental stage and independent of the N fertilization conditions. Moreover, compared with the V stage, the expression of far fewer genes appeared to be altered at the M stage (Fig. 5).

Integrative view on the differences in accumulation of metabolites, proteins, and transcripts

It was first examined whether there was any correspondence in terms of biological function between protein and transcripts accumulating at the two plant developmental stages, when N was either available or deficient. Only limited correspondence was found between the two sets of data, suggesting that changes in transcript and protein accumulation occur independently. These correspondences are highlighted in Supplementary Tables S3–S6 at *JXB* online. The biological processes involved are mostly related to various developmental processes and to some defence mechanisms.

Up to now, there have been very few attempts to obtain an integrated view of the interactions occurring between the biological components identified simultaneously in three ‘omics’ studies (Hirai *et al.*, 2004; Nielsen and Olivier, 2005; Barros *et al.*, 2010). Moreover, in most cases these integrative studies did not take into account the spatial (organs and tissues) and temporal control (developmental stage) of these biological processes, occurring from gene expression to metabolite accumulation. It will be also necessary to take into account diurnal changes in the accumulation of both metabolites and transcripts in order to obtain a more dynamic picture of their importance, when plant metabolic activity is modified during a diurnal cycle (Usadel *et al.*, 2009).

In the present study, a preliminary attempt was made to integrate the three ‘omics’ levels and the impact of long-term N deficiency at two key stages of plant development. It was decided to base the experimental design on long-term N deficiency, instead of short-term N starvation, or a short period of N resupply, in order to obtain a steady-state picture of mRNA transcript, protein, and metabolite accumulation. Such a picture can provide important information on plant responses and thus the physiological adaptation to the prolonged N deficiency stress that may be encountered under agronomic conditions. Irrespective of their biological function, the quantitative differences observed in transcript, protein, and metabolite accumulation in relation to the plant developmental stage are presented in Table 3. In addition, the relative importance of these biological functions in terms of number of metabolites, proteins, and transcripts exhibiting differences within common functional categories across the three ‘omics’ studies is presented in Fig. 5. One can observe that the most important differences within the repertoire of the main plant biological processes were generally observed at the transcript accumulation level, whatever the plant developmental stage. This means that the response of a plant to long-term N deprivation affects primarily the expression of genes encoding enzymes involved in metabolic pathways and also a variety of other proteins playing a key role in development, signalling, and transcriptional regulation processes.

Fewer variations in functional diversity were observed at the metabolomic level, although the decrease in metabolite content was the main consequence of N deficiency, particularly at the V stage (Table 3, Fig. 5). This is probably because the end-products of the different metabolic pathways, independent of their regulation, mostly originate from C and N metabolism. Moreover, one has to consider that the same metabolite may be involved

in multiple metabolic pathways. Nevertheless, it is clear that the biological process that is the most strongly affected when N is limiting is in a broad sense C metabolism, including photosynthesis, sugar, and organic acid biosynthesis. It can be seen clearly that these various biological processes related to the acquisition and distribution of C are either up- or down-regulated at the three ‘omic’ levels in terms of both intensity and metabolic specificity, depending mostly on the plant developmental stage. The finding that across the three ‘omics’ experiments, cell wall component biosynthesis as well as stress and defence mechanisms were altered fits with the observation that C metabolism, lignin biosynthesis, and stress-responsive elements are coordinately regulated (Dauw *et al.*, 2007). Interestingly, at both the transcriptome and proteome level, a number of stress-responsive elements and plant defence mechanisms were up-regulated when N was limiting. This observation suggests that the lack of N induces a plant response similar to that occurring in other biotic and abiotic stresses (Mittler and Blumwald, 2010). Also, it is not surprising to find that the perturbations of N metabolism are mostly detected at the level of metabolite accumulation, since carbon skeletons are required to synthesize most organic N molecules (Hirel *et al.*, 2001) and taking into account that C metabolism is the metabolic process that is the most strongly perturbed when N is limiting.

Although a diversity similar to that found at the transcriptome level would have been logically expected, differences in the protein profile were relatively minor and involved a limited number of plant biological functions. This is probably because a significant number of low-abundant structural or regulatory proteins cannot be detected using 2-D gel electrophoresis in comparison with the microarrays containing most of the maize transcripts (Fig. 5). It is also possible that the plant response at the translational level is buffered somehow in comparison with gene transcription, metabolic fluxes, and metabolite accumulation, thus suggesting that there is a relatively stable pool of proteins that is not modified under N-deficient conditions. Moreover, the plant response to N deficiency may involve a number of post-translational protein modifications that were not investigated in the present study.

Conclusion and perspectives

Nowadays, increasing amounts of ‘omics’ data related to the response of both model and crop plants to short-term and long-term N-deficiency are available (Urbanczyck-Wochniak and Fernie, 2005; Rubin *et al.*, 2009; Broyard *et al.*, 2010; Fait *et al.*, 2011; Krapp *et al.*, 2011; Kusano *et al.*, 2011). However, despite this wealth of information, linking these data to a plant phenotype in terms of plant growth, development, and yield remains a challenge. This is mainly because, as shown in the present study and a number of recent investigations (Hirai *et al.*, 2004; Kant *et al.*, 2011), when examining individually the biological systems involved, there is only a single level of complexity from gene expression to protein and metabolite accumulation which increases when their interactions are considered (Gutiérrez *et al.*, 2007). Moreover, there is often very little direct correspondence between differences in the accumulation of metabolites, proteins, and transcripts (for a review, see Fernie and Stitt, 2012), due to the complexity inherent in biological

Table 3. Overview of the main changes occurring in a maize leaf at the metabolome, proteome and transcriptome level. Biological functions were classified into functional categories for each of the three ‘omics’. Plants were grown under low (N⁻) or high (N⁺) fertilization conditions and harvested at the vegetative (V) or maturity (M) stage. The functional category that was the most strongly altered when considering the number of different metabolites, proteins, and mRNA transcripts is indicated in upper case letters. The different functional categories were obtained from the different analyses presented in Supplementary Tables S1–S6 at JXB online.

Transcriptome	Proteome	Metabolome	Transcriptome	Proteome	Metabolome
Lower in N ⁻			Higher in N ⁻		
Vegetative					
C assimilation	C assimilation	OA METABOLISM	C metabolism	C assimilation	OA metabolism
C metabolism		Sugar metabolism		GLYCOLYSIS	SUGAR METABOLISM
Glycolysis				Energy transfer	
PHOTOSYNTHESIS					
Cell wall		Lignin biosynthesis	Cell wall		Lignin biosynthesis
N assimilation		N METABOLISM			
METABOLISM			Metabolism		
Defence	Defence		Defence	Defence	
Stress	Stress		Stress		
Signalling			SIGNALLING		
Proteolysis	Proteolysis		Proteolysis		
			Development		
Transport			Transport		
Translation	TRANSLATION		Translation		
Transcription			TRANSCRIPTION		
Protein folding					
Maturity					
Photosynthesis	PHOTOSYNTHESIS	OA metabolism	Photosynthesis	Photosynthesis	OA METABOLISM
		Sugar metabolism			Sugar metabolism
		N metabolism			
Metabolism			Metabolism		
			Lipid metabolism		
Defence					
	Stress		Stress		
SIGNALLING	Signalling		Signalling		
	Proteolysis				
	Development				
	Transport				
	Protein folding				
			NUCLEOSOME		

networks and to the fact that both tissue and cellular compartmentation within different organs are generally not taken into account. Nevertheless, the most interesting finding in the present study is that depending on the level of N nutrition, a major biological function such as C assimilation and several metabolic pathways and stress-responsive and regulatory elements linked to C utilization share common characteristics across at least two or even three of the ‘omics’ considered (Fig. 5).

In spite of this, there are some limitations in the interpretation of the present metabolic data, since they generally did not reflect the flux of compounds going through a pathway, which needs to be considered at both the organ and tissue levels, during plant growth and development (Fernie and Stitt, 2012). Ideally, metabolomic studies should be completed with fluxomic studies using labelled molecules (Mesnard and Ratcliffe, 2005; Kruger and Ratcliffe, 2012) performed on individual organs harvested at different stages of plant development and over a diurnal cycle. Using the data gathered from the various ‘omics’, construction of large-scale metabolic models will certainly constitute a valuable tool for determining which steps across the ‘omics’ are

limiting during plant growth and development (Gomes de Oliveira Dal’Molin *et al.*, 2010). The recent achievements in quantitative genetic studies, which were able, in some cases, to identify key elements involved in plant productivity with respect to N metabolism and N availability (Hirel *et al.*, 2007; 2011), indicate that it is possible to link a phenotype to DNA markers and thus to a gene or a set of genes. Even in the above approaches, which are similar to those performed in the present study, only a snapshot of the plant metabolic status was considered by measuring metabolites or enzyme activities in a single organ; the validation of the underlying candidate genes was in some cases successfully achieved (Prioul *et al.*, 1999; Martin *et al.*, 2006). This type of ‘static’ approach has allowed the partial bypass the complexity of the biological systems involved, by making a direct jump from a phenotypic trait to putative candidate genes, whatever the plant developmental stage and the time of harvest. Therefore, combining ‘omics’ studies in an integrated manner (Zhang *et al.*, 2010; Stitt and Fernie, 2012) and investigations aimed at identifying the genetic basis of plant NUE should help to identify the limiting steps involved in the control of NUE.

Furthermore, the N-responsive genes identified in transcriptome studies, like those performed in the present investigation, can be placed on a maize genetic map so that co-localizations with quantitative trait loci (QTLs) for yield and its components, or QTLs for NUE can be identified in both vegetative and reproductive organs (Limami *et al.*, 2001; Gallais *et al.*, 2004; Cañas *et al.*, 2012). Validation of candidate genes can then be undertaken using transgenic technologies (forward genetics), or mutagenesis (reverse genetics), or by studying the relationship between allelic polymorphisms and the trait of interest (association genetics), at either a single gene or a genome-wide level. Positional cloning is another alternative strategy that can be used to focus on the chromosomal region controlling the trait of interest, which may ultimately allow access to a single gene (for a review, see Hirel *et al.*, 2011). As a complement to these two approaches, new gene, protein, and metabolite markers can be identified thanks to the knowledge gained from the 'omics' studies performed on different genotypes under various environmental conditions (Yang *et al.*, 2011). Ultimately these markers can be used for the production of diagnostic tools, which will allow the monitoring of their levels of expression, regardless of the genetic background or of the environmental effects (Kichey *et al.*, 2006; Barros *et al.*, 2010; Hirel *et al.*, 2011; Yang *et al.*, 2011). These diagnostic tools could be directly utilized by breeders to screen for the best performing genotypes. Consequently, metabolomics in combination with the other 'omics' could also be applied to plants by providing a snapshot of their metabolic state under various environmental conditions (Barros *et al.*, 2010), or when they are subjected to biotic or abiotic stresses in order to adapt their fitness to adverse environmental conditions and in turn stabilize or increase yield.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Metabolites exhibiting significant changes in their concentration at the V stage.

Table S2. Metabolites exhibiting significant changes in their concentration at the M stage.

Table S3. Proteins exhibiting significant variation in their amount at the V stage.

Table S4. Proteins exhibiting significant variation in their amount at the M stage.

Table S5a. Transcripts exhibiting a significant decrease under non-limiting N feeding conditions at the V stage.

Table S5b. Transcripts exhibiting a significant increase under N-limiting conditions at the V stage.

Table S6. Transcripts exhibiting a significant increase and decrease in their amount at the M stage.

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